E-ISSN:2456-3110

Research Article

Mercurial Preparation

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Journal of Ayurveda and Integrated Medical Sciences

2025 Volume 10 Number 9 September

Publisher

Maharshi Charaka Ayurveda

www.maharshicharaka.in

Evaluation of the Anti-Carcinogenic and Anti-Inflammatory Effects of Lokanatha Rasa: A Mercurial Preparation

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DOI:10.21760/jaims.10.9.7

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Rasa Shastra is a branch of Ayurveda pharmaceutics that focusses on mercury-based preparations known as Rasaushadies. Lokanatha Rasa (LKN) is a Rasaushadie mentioned Rasasendra Sara Sangrahya, and is especially indicated for Liver and spleen disorders. It contains Kajjali (Parada and Gandhaka), and incinerated forms (Bhasma) of Abhra, Lauha, Thamra and Varatika. No scientific research has been conducted regarding LKN. This study aims on the evaluation of the invitro anticarcinogenic activity of LKN using HepG2 cancer cell line and to evaluate the anti-inflammatory activity of it using the HRBC (Human Red Blood CELL) membrane stabilizing method. Cell viability of different concentrations of LKN on Hep G2 cell line was studied using MTT assay. Therefore, prevention of hypotonicity induced HRBC membrane lysis was taken as an invitro measure of anti-inflammatory activity of the drug. The inhibition percentage followed an exponential increase beyond 2 mg/mL, suggesting a threshold concentration at which LKN exerts significant cytotoxic effects. The MTT assay was directly proportional to its concentration. At the lowest tested concentration (0.0625 mg/mL) the % of inhibition was 27.73% and the highest concentration of LKN i.e. 1000mg/mL% of inhibition was 67.01. Results reveal that LKN possesses anti-inflammatory activity, though less than Diclofenac sodium.

Keywords: Lokanatha Rasa, HepG2 cell line, HRBC membrane stabilizing, MTT assay

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How to Cite this Article

SKMK Herapathdeniya, PA Paranagama, JGS Ranasinghe, Evaluation of the Anti-Carcinogenic and Anti-Inflammatory Effects of Lokanatha Rasa: A Mercurial Preparation. J Ayu Int Med Sci. 2025;10(9):40-45.

Available From

https://jaims.in/jaims/article/view/4749/

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Manuscript Received	Review Round 1	Review Round 2	Review Round 3	Accepted
2025-07-15	2025-07-28	2025-08-08	2025-08-18	2025-08-27

Conflict of Interest	Funding	Ethical Approval	Plagiarism X-checker	Note
None	Nil	Not required	10.32	

Introduction

Rasa Shasta is a principal branch in Ayurveda pharmaceutics, with Mercury being its major element. Preparations made using Mercury are collectively known as *Rasaushadies.*[1] *Lokanatha Rasa* (LKN) is one such mercurial formulation mentioned in the classical Rasa Shastra text *Rasendrasara Sangraha* (Chapter 3, Page number 559) and is considered to be effective for the management of liver and spleen disorders.[2] But there is no scientific research conducted regarding the efficacy of LKN for liver disorders. This study aims the evaluation of the invitro anti carcinogenic effect LKN on HepG2 cell line and to evaluate its anti-inflammatory effect, with the use of Human Red Blood Cell (HRBC) membrane stabilizing assay. Hepatocellular Carcinoma (HCC) is the most prevalent type of cancer in the liver, characterized by uncontrolled proliferation of hepatocytes and high mortality rates. Globally, it is the 4th most common cancer and 2nd most leading cause of cancer related deaths.[3]

Despite advancements in chemotherapy, drug resistance and toxicity remain major challenges in HCC treatment. In Rasa Shastra, several mercurial and mineral based formulations have been traditionally used for the management of tumors and malignancies. Certain *Rasaushadies* (herbo-mineral formulations) are believed to inhibit tumor growth and exert cytotoxic effects on cancer cells, making them potential candidates for anticancer research.[4] The Human Hepatocellular Carcinoma (HepG2) cell lines serve as an in vitro model for studying the cytotoxic effects of compounds on liver cancer. These immortalized cell lines are widely used due to their stable genetic profile, easy maintenance, and metabolic enzyme activity that remains consistent during cultivation.[5] In this study, different concentrations of *Lokanatha Rasa* (LKN) were evaluated for anti - carcinogenic activity using the MTT assay against HepG2 cell lines, assessing cell viability and cytotoxicity.

Inflammation is the body's physiological response by the living tissues to injury, infection or irritation.[6] The exposure of RBC to some injurious substances like heat, hypotonic medium, Methyl salicylate, or Phenylhydrazine its membrane becomes prone to lysis due to haemolysis and oxidation of Hemoglobin.[7]

Due to uncontrolled, persistent chronic inflammation serious health problems such as Heart attacks, Rheumatoid arthritis and also septic shocks could occur.

Human Red Blood Cell Membranes are analogues to the lysosomal membranes,[8] hence the prevention of hypotonicity induced HRBC membrane lysis was taken as an invitro measure of anti-inflammatory activity of the drug.

Objectives

1. Evaluation of the in vitro anti-carcinogenic activity of *Lokanatha Rasa* using the MTT assay on HepG2 cell lines.

2. An assessment of the anti-inflammatory potential of LKN using HRBC membrane stability assay, via the ability to prevent any hypotonicity induced hemolysis.

Materials and Methods

Ethical clearance: Obtained from the Ethics Review Committee of Bandaranaike Memorial Ayurveda Research Institute (ERC No - BMARI/2023/003)

Assess anti carcinogenic effect by using Hep G2 cell line study

Cell line and storage

HepG2 cell line (ECACC general collection; Cat No. 85011430) was used for this study. The cells were cryopreserved in liquid Nitrogen and thawed for experimental use.

Establishing and maintenance of HepG2 cell line

HepG2 cells were expanded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotics (Penicillin 50 IU/mL) and Streptomycin (50 µg/mL).

The cells were maintained at 37°C in a CO_2 incubator (Memmert, Germany) at 80% - 88% humidity and 5% CO_2. Culture medium was changed every third day to maintain optimal cell growth conditions. Once the cells reached 70% confluency, they were sub-cultured at a seeding density of 2 x 10^5 cells per sterile 25 cm^2 cell culture flask.

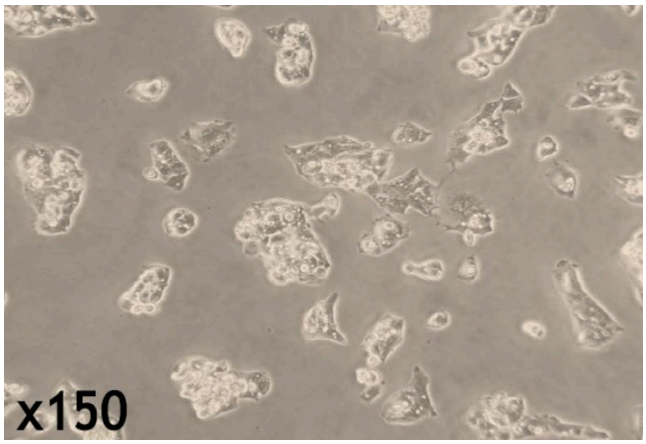

Plate 1: Images of growing clusters of HepG2 adherent cell colonies on the bottom of the cultured plates captured under inverted microscopy. (x150 magnification)

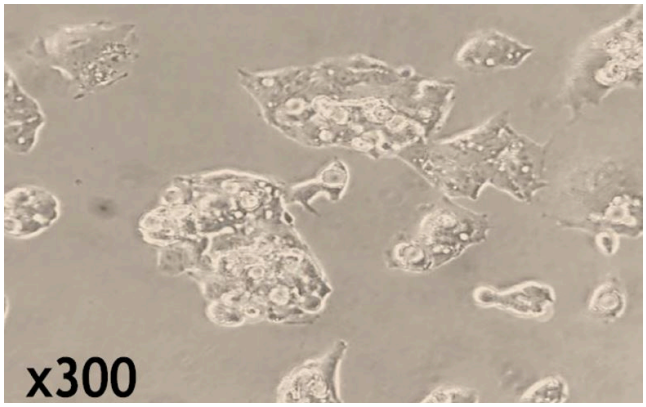

Plate 2: Images of growing clusters of HepG2 adherent cell colonies on the bottom of the cultured plates captured under inverted microscopy. (x300 magnification)

Cell Viability

Cell viability assay was conducted by using MTT assay

MTT [3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazolium bromide] assay

MTT assay was conducted according to the method described by Cardile et, al: (2004).**[9]** The viability of the cells was assessed by MTT assay which is based on the reaction of MTT by the mitochondrial dehydrogenase of intact cells to a purple color formazan product. Cells were plated in a 96 well plate (Corning, USA) at a seeding density of 5 x 10^3 cells/well and incubated at 37°C in a CO_2 incubator for 24 hours. Different concentrations of the LKN (0.08 mg/mL, 0.1 mg/mL, 0.8 mg/mL, 1.2 mg/mL, 1.6 mg/mL, 2. mg/mL, 2.4 mg/mL, 2.8mg/mL, 3.2mg/mL, 3.6 mg/mL, 4 mg/mL, And 5mg/mL) prepared by diluting the preparation in DMEM (Sigma Aldrich, USA) culture medium. After 24 hours, remaining medium in each well was aspirated, and cells were treated for 24 hours with prepared LKN concentrations in triplicate at 100 µl/well. Three wells were treated with culture medium was used as the normal control, while anti-cancer drug, Cisplatin (0.2mg/ml) was used as the positive control.

After the 48 hours of treatment, 20 µl of MTT solution (5mg/mL) was dispensed into each well and incubated for 4 hours at 37 °C in a CO_2 incubator. Then the culture medium was aspirated, and 100 µl of Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, USA) was dispensed into each well. After incubating the plate at RT with shaking, optical density (OD) measurements were obtained using a microplate reader (BIO-RAD, Model 680, USA) using a 540 nm filter. Percentage inhibition (% Inhibition) was calculated using the following equation: findings confirm that LKN exhibits moderate cytotoxicity activity against HepG2 cells, supporting its potential as an anticarcinogenic agent.

% Inhibition = (OD Control – OD Test) / OD Control

Accessing the anti-inflammatory activity of LKN by using HRBCM stabilizing assay.

Preparation of LKN extract

Initially 100 mL two conical flasks were cleaned using acetone (Sigma Aldrich USA). 20g of drug sample (LKN)was placed in each conical flask separately. 50mL of methanol (Sigma Aldrich USA) solution was added to each conical flask. Following that the mixtures were shaked and allowed to stand at room temperature for 24 hours with occasional shaking. Next, the extracted solution was filtered using Buchner funnel with Whatman No 1 filter paper. The filtrate was then collected to the clean flask. The filtrate was concentrated using a rotary evaporator (IKA-RV,10 digital, Germany) to remove the solvent. The final extract of crude was prepared using nitrogen purge techniques. Once the crude extract was prepared it was stored in a clean small culture bottle and placed inside the refrigerator

Ethical Approval

Ethical clearance for this study was obtained from the Ethics Review Committee of Bandaranayake Memorial Ayurveda Research Institute (ERC No - BMARI/2023/003)

Reagents

1. Alsever solution pH 6.4 (2% Dextrose, 0.8% Sodium citrate, 0.05% Citric acid, 0.42% Nacl in water)
2. Iso saline (0.85% pH 7.2)
3. Phosphate buffer (0.15M, pH 7.4)
4. Hyposaline (0.36%) used in this study were at analytical grade and purchased from Sigma-Aldrich, USA)

All chemicals

Procedure

3.0 ml of fresh whole blood was collected from healthy volunteers who had not taken any Non-Steroidal Anti-inflammatory Drugs (NSADS) within the preceding 24 hours. Then the collected blood samples were mixed with equal volume of sterilized Alsever's solution. This mixture was then centrifuged at 3000 rpm for 10 minutes, and the packed cells were washed with Iso saline (0.85%, pH 7.2). This process was repeated 3 times and the thick blood cells out of it were separated. 9mL of iso saline was added to the 1mL of separated red blood cells and a 10% v/v solution was prepared.**[10],[11]** This was called HRBC suspension.

The assay mixture contained 1.0ml of phosphate buffer, (pH 7.4,0.15M) 2.0ml of hyposaline, (0.36%) 0.5ml HRBC suspension (10%V/V) and 1.0ml 0f various concentrations of LKN (1.0, 0.5, 0.25, 0.125, 0.0625 mg/ml)

For the standard 1.0 ml diclofenac solution (1.0,0.5,0.25,0.125,0.0625 mg/ml) was used in place of LKN.

For the solution 2.0ml of double distilled water was added.

The mixtures were incubated at 37°C for 30 mins and centrifuged at 3000 rpm for 5minutes.

The absorbance of the supernatant solution was measured at 560nm.

The percentage of HRBC membrane stabilization was calculated by assuming the hemolysis produced in distilled water (control) as 100%.**[12]** The percentage of HRBC membrane stabilizing was calculated using the following formula.

Percentage of hemolysis = (Optical density of the sample X 100) / Optical density (Control)

Percentage of membrane stabilization

% of protection = 100 - Optical density of test sample / Optical density (Control)

Results

Cytotoxicity of LKN on Hepatocellular carcinoma cell line

A wide range of concentrations of the LKN was tested on HepG2 cells to investigate the cytotoxic ranges and to calculate the IC50 value of the preparation using the MTT assay.

HepG2 cells were treated with a range of concentrations (0.08mg/mL – 5 mg/mL) of LKN

Table 1 presents the percentage of the inhibition values of different concentrations of the LKN on Hep G2 cells

Table 1: % inhibition values of different concentrations of the LKN on HepG2 cells

Test concentrations (mg/ml)	% inhibition	SD
PC (Cisplatin)	98.7%	0.9%
0.08	-4.8%	6.6%
0.1	12.2%	1.4%
0.8	25.9%	7.1%
1.2	41.4%	14.9%
1.6	48.8%	4.0%
2	70.4%	2.8%
2.4	81.8%	8.1%
2.8	86.2%	2.1%
3.2	96.9%	1.7%
3.6	102.4%	2.9%
4	96.7%	13.0%
4.5	100.0%	7.0%
5	100.1%	7.2%

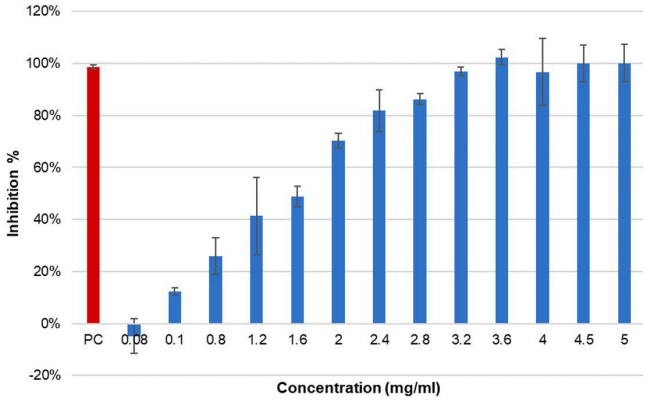

Figure 1: Effect of LKN on HepG2 viability.

LKN exhibited a dose – dependent inhibitory effect on HepG2 cell viability.

At concentrations above 2 mg/mL, more than 50% cytotoxicity was observed, indicating strong cytotoxic response (Figure 1). Half – maximal inhibitory concentration (IC_{50}) for LKN was calculated as 1.6 mg/mL, indicating concentration required to reduce HepG2 cell viability by 50%. The IC_{50} of standard chemotherapy drug Cisplatin was 0.13 mg/mL, which is significantly lower than LKN (1.6 mg/mL). This indicates that LKN requires higher concentration to achieve similar cytotoxicity levels compared to Cisplatin. Inhibition percentage followed an exponential increase beyond 2 mg/mL, suggesting threshold concentration at which LKN exerts significant cytotoxic effects. Observed reduction in cell viability suggests that LKN induces cytotoxicity in HepG2 cells, which may be attributed to apoptotic or metabolic inhibitory mechanisms. Microscopic observations revealed morphological alterations in HepG2 cells treated with higher concentrations of LKN, including cell shrinkage, membrane blebbing, & detachment from culture plate, all of which are indicative of cell death pathways. These findings confirm that LKN exhibits moderate cytotoxicity activity against HepG2 cells, supporting its potential as an anticarcinogenic agent.

The anti-inflammatory activity of LKN was tested by HRBC stabilization method and results were mentioned in above Table 1 and Figure 1.

Anti-inflammatory assay

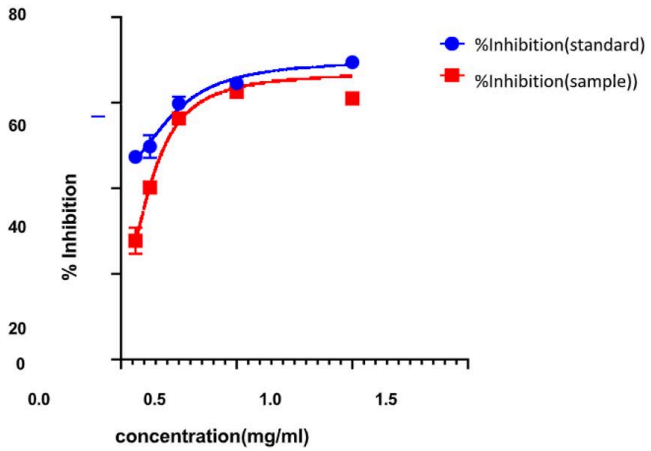

Figure 2: Anti-inflammatory effect of LKN under different concentrations

Discussion

HepG2 is widely utilized HCC cell line, originally derived from liver tissue of 15 – year – old Caucasian male with well - differentiated Hepatocellular Carcinoma.

Due to its stable genetic profile, metabolic activity, and ease of maintenance, it serves as a suitable in vitro model for studying drug metabolism, cytotoxicity, and anti – cancer efficacy.**[13]** HepG2 cells exhibit adherent, epithelial – like morphology, growing in monolayers and small aggregates, making them ideal for drug screening and mechanistic studies of hepatic carcinogenesis.

In the present study, HepG2 cells were selected to assess the anti-cancer activity of LKN. The cytotoxic effects were evaluated using the MTT assay, a standard method for assessing cell viability and proliferation inhibition. The results revealed that LKN exhibited a progressive cytotoxic effect against HepG2 cells. At concentrations above 2 mg/mL, more than 50% cytotoxicity was observed, indicating a strong inhibitory effect. The half - maximal inhibitory concentration (IC_{50}) for LKN was 1.6 mg/mL, suggesting its moderate anti-cancer potential. The IC_{50} of Cisplatin (0.13 mg/mL) was significantly lower than LKN (1.6 mg/mL), indicating that LKN requires a higher concentration to achieve similar cytotoxicity. This suggests that while LKN shows anti-cancer activity, it is less potent than conventional chemotherapy drugs like Cisplatin. Beyond 2 mg/mL, the inhibition percentage increased exponentially, indicating a threshold concentration at which LKN exerts maximum cytotoxic effects.

The anti-cancer potential of LKN may be attributed to apoptotic induction, oxidative stress generation, or metabolic inhibition, although further studies such as flow cytometry for apoptosis markers, reactive oxygen species (ROS) quantification, and gene expression analysis are required to elucidate its mechanism of action.

The anti-inflammatory activity of LK was tested by HRBC stabilizing assay was directly proportional to its concentration. The values being in the lowest concentration of 0.0625 mg/mL % of inhibition was 27.73 and the highest concentration of LKN i.e. 1000 mg/mL% of inhibition was 67.01

When LKN compared with the standard Diclofenac the HRBC stabilization was higher in Diclofenac sodium. When the data were subjected to two-way ANOVA the values were highly significant at $p<0.001$ level. Results reveal that LKN possesses anti-inflammatory activity, but it is lower than Diclofenac sodium.

Conclusion

The findings of this study demonstrate that LKN exerts noticeable cytotoxic and apoptotic effects of HepG2 cells, providing scientific validation of its anticancer potential. The observed dose - dependent cytotoxicity of LKN suggests its possible role as a complementary or alternative therapeutic agent in the treatment for HCC. Although LKN exhibited a higher IC_{50} than Cisplatin, its traditional processing techniques, including *Shodhana, Marana,* and *Amritikarana,* may contribute to reduced systemic toxicity while retaining its therapeutic efficacy. These findings suggest that *Rasa Shastra* mercurial formulations like LKN may hold promise for integrative cancer therapy. The results which are obtained from the ant inflammatory study demonstrated that LKN can significantly and dose dependently inhibit lysis of human red blood cell membrane, hence stabilizing the human red blood cell (HRBC) membranes. Given that the destabilization of membranes can occur due to chronic liver diseases, the effect of LKN underscores the potential benefit of it associated with the management of anti-inflammatory action related to hepatic diseases.

Source of support: Purchase of chemicals and cell line funded by United Nations Industrial Development Organization (UNIDO), Geneva; Government of Switzerland (via the Ministry of Environment, Sri Lanka).

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